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All-*trans*-Retinoic Acid Represses Obesity and Insulin Resistance by Activating both Peroxisome Proliferation-Activated Receptor β/δ and Retinoic Acid Receptor▽†

Daniel C. Berry and Noa Noy*

Department of Pharmacology and Department of Nutrition, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

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Many biological activities of all-*trans*-retinoic acid (RA) are mediated by the ligand-activated transcription factors termed retinoic acid receptors (RARs), but this hormone can also activate the nuclear receptor peroxisome proliferation-activated receptor β/δ (PPARβ/δ). We show here that adipocyte differentiation is accompanied by a shift in RA signaling which, in mature adipocytes, allows RA to activate both RARs and PPARβ/δ, thereby enhancing lipolysis and depleting lipid stores. In vivo studies using a dietary-induced mouse model of obesity indicated that onset of obesity is accompanied by downregulation of adipose PPARβ/δ expression and activity. RA treatment of obese mice induced expression of PPARβ/δ and RAR target genes involved in regulation of lipid homeostasis, leading to weight loss and improved insulin responsiveness. RA treatment also restored adipose PPARβ/δ expression. The data indicate that suppression of obesity and insulin resistance by RA is largely mediated by PPARβ/δ and is further enhanced by activation of RARs. By targeting two nuclear receptors, RA may be a uniquely efficacious agent in the therapy and prevention of the metabolic syndrome.

The vitamin A metabolite all-*trans*-retinoic acid (RA) regulates gene expression by activating specific members of the superfamily of transcription factors known as nuclear hormone receptors. It has long been established that many biological activities of RA are mediated by retinoic acid receptors (RARα, RARβ, and RARγ) (7, 23). However, recent observations revealed that, in addition to activating RARs, RA can also serve as a ligand for the nuclear receptor peroxisome proliferation-activated receptor β/δ (PPARβ/δ) (32, 33, 37). Like other subclass 1 nuclear receptors, RARs and PPARβ/δ function as heterodimers with the retinoid X receptor (RXR), and they regulate transcription by binding to regulatory regions of target genes harboring response elements (RE) composed of two direct repeats of the motif 5′-PuG(G/T)TCA. RXR-RAR heterodimers bind to RE in which the repeats are spaced by 2 or 5 bp (DR-2, DR-5) (21). RE for RXR-PPAR heterodimers are comprised of a DR-1 containing an extended 5′-half site, an imperfect core DR1, and an adenine as the spacing nucleotide (8). Ligand binding by these heterodimers results in receptor activation and usually enhances the rate of transcription of target genes.

The RA receptors, RARs and PPARβ/δ, are often coexpressed in cells, and it has been demonstrated that the partitioning of the hormone between them is regulated by two members of the family of intracellular lipid-binding proteins (iLBP). The iLBPs are small (~15 kDa) cytosolic proteins that include cellular retinol-binding proteins, cellular RA-binding proteins (CRABP), and nine isotypes of fatty acid-binding proteins (FABP) (4, 28). Within this family, CRABP-II specifically cooperates with RARs, and FABP5 (mal1, eFABP, and K-FABP) functions in conjunction with PPARβ/δ. Like other iLBPs, CRABP-II and FABP5 are cytosolic in the absence of ligand. However, upon binding of RA, these proteins mobilize to the nucleus, where they associate with RAR and PPARβ/δ, respectively, to form complexes through which RA is "channeled" from a binding protein to a cognate receptor. CRABP-II delivers RA to RARs (5, 10, 24, 36), while FABP5 shuttles the hormone to PPARβ/δ (32, 39). CRABP-II and FABP5 thus facilitate the ligation and enhance the transcriptional activities of RARs and PPARβ/δ, respectively (5, 39). Consequently, the relative expression levels of CRABP-II and FABP5 in different cells determine the nature of the receptor that is activated by RA, the spectrum of RA-inducible genes, and the biological responses to the hormone (32). For example, we recently showed that, in mammary carcinomas with a high CRABP-II/FABP5 ratio, RA activates RARs, thereby inducing the expression of genes that trigger cell cycle arrest and apoptosis and inhibiting cell growth. In contrast, in mammary carcinomas that express a low CRABP-II/FABP5 ratio, RA treatment upregulates PPARβ/δ target genes involved in mitogenic and antiapoptotic responses and thus enhances proliferation (33).

Among the biological functions of PPARβ/δ, of special interest is the involvement of this receptor in regulation of energy balance. It has been reported in regard to this that the repertoire of direct targets for PPARβ/δ includes genes involved in lipid and glucose metabolism and that activation of this receptor increases lipid catabolism in skeletal muscle and adipose tissue, prevents the development of obesity, improves

* Corresponding author. Mailing address: Department of Pharmacology, Case Western Reserve University, 10900 Euclid Ave., SOM W333, Cleveland, OH 44106. Phone: (216) 368-0302. Fax: (216) 368-1300. E-mail: nxn51@case.edu.

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insulin sensitivity in obesity-prone mouse models, and enhances exercise endurance (3, 6, 18, 26, 41, 42). Consequently, it has been proposed that PPARβ/δ agonists may be efficacious agents in the treatment of the metabolic syndrome (3). The observations that RA functions as a PPARβ/δ ligand raise the intriguing possibility that this hormone may play important roles in regulation of sugar and lipid metabolism and disposition. In addition to PPARβ/δ, the classical RARs may also be involved in regulating lipid and energy homeostasis. For example, available information suggests that uncoupling protein 1 (UCP1), a protein that mediates energy dissipation, and apolipoprotein A1 (apo A1), which is involved in plasma transport of cholesterol and other lipids, may be regulated by both PPAR and RARs (14, 30, 31).

We thus set out to investigate whether signaling through PPARβ/δ and/or RARs underlie the involvement of RA in regulation of energy homeostasis. Using cultured adipocytes and a high-fat/high-carbohydrate diet-induced mouse model of obesity, we show that, in mature adipocytes, RA signals through both RARs and PPARβ/δ to induce the expression of multiple genes involved in regulation of energy homeostasis and insulin responses. We show further that administration of RA to obese mice leads to the loss of fat mass and improved glucose tolerance and that these effects can be traced to activation of PPARβ/δ and RARs in adipose tissue, muscle, and liver. The observations suggest that, due to its ability to activate two nuclear receptors, RA is a uniquely effective agent in suppressing adiposity and insulin resistance.

MATERIALS AND METHODS

Reagents. Antibodies against CRABP-II were provided by Cecile Rochette-Egly (13). Antibodies against FABP5 were from R&D Systems. Antibodies to glycogen synthase kinase 3β (GSK3β), phosphorylated GSK3α/β, Akt1, and phosphorylated Akt1 were from Cell Signaling. Antibodies against RARα, RARβ, and succinate dehydrogenase (SDH) were from Santa Cruz Biotechnologies. Antibodies against RARγ, β-tubulin, and PPARβ/δ were from Affinity BioReagents, Sigma Aldrich, and Abcam, respectively. Anti-mouse and anti-rabbit immunoglobulin horseradish peroxidase-conjugated antibodies were from Bio-Rad Laboratories. Anti-rabbit immunoglobulin G conjugated to Alexa Fluor was from Invitrogen. RA was purchased from Calbiochem, RA pellets were obtained from Innovative Research of America. 4-[(*E*)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) and GW0742 were purchased from Biomol International and Toronto Research Diagnostics, Inc., respectively.

Biochemical analyses. Lipolysis and triglyceride accumulation were assessed by using Zen Bio lipolysis and triglyceride accumulation kits. Cell and tissue extractions and immunoblot analyses were performed as previously described (32). Densitometries for immunoblots were determined by using the ImageJ program (National Institutes of Health [NIH]). For quantitative real-time PCR analysis (Q-PCR), RNA was extracted by using an RNeasy minikit (catalog no. 74104), RNeasy lipid tissue minikit (catalog no. 74804), and RNeasy fibrous tissue minikit (catalog no. 74704), and cDNA was generated by using GeneAmp RNA PCR (Applied Biosystems). Q-PCR was carried out by using TaqMan chemistry and Assays-on-Demand probes (Applied Biosystems) for CRABP-II (Mm00801691_m1), FABP5 (Mm00783731_s1), PPARβ/δ (Mm01305434_m1), adipose differentiation-related protein (ADRP; Mm00475794_m1), ANGPTL4 (Mm001278813_m1), Cyp26a (Mm00514486_m1) UCP1 (Mm00494069_m1), UCP3 (Mm00494074_m1), aldehyde dehydrogenase 9 (ALDH9; Mm00487200_m1), 3-phosphoinositide-dependent protein kinase 1 (PDK1; Mm00554306_m1), CPT1B (Mm00487200_m1), GluT4 (Mm00436615_m1), RARα (Mm00436264_m1), RARβ (Mm01319680_m1), RARγ (Mm00441083_m1), hormone sensitive lipase (HSL; Mm00495359_m1), hapo A1 (Hs00163641_m1), and mapo A1 (Mm00437569_m1). 18s rRNA (4352930E) was used as a control.

3T3-L1 cell differentiation. 3T3-L1 fibroblasts were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% calf serum. Cells were allowed to grow for 2 days past confluence, and differentiation medium was added (DMEM supplemented with 10% fetal bovine serum [FBS], 10 μg of insulin [Sigma Aldrich]/ml, 0.5 mM IBMX [3-isobutyl-1-methylxanthine; Sigma Aldrich], and 0.25 mM dexamethasone [ThermoFisher]). Three days later, the medium was replaced with DMEM supplemented with 10% FBS, and the cells were allowed to continue to differentiate for an additional 4 days. Differentiation was determined by Oil red O staining and by monitoring the expression of FABP-4.

Lentiviruses harboring shRNA. Mouse FABP5 short hairpin RNA (shRNA; TRCN0000011894) and mouse PPARβ/δ shRNA (TRCN0000026045) were obtained from Open Biosystems, and viruses were produced according to the protocol of the manufacturer. Viruses were harvested and cells infected twice with a 24-h interval. Experiments were carried out 5 days later.

Mouse studies were performed by the Mouse Metabolic Phenotyping Center of the Case Western Reserve University. C57BL/6Ntac mice (Taconic) were placed on a high-fat/high-sucrose diet (HFHS research diet D12331) for 16 weeks prior to experimentation. Lean mice were fed chow diet (LabDiet 5P76 Irradiated Isopro RMH 3000 [Prolab, St. Louis, MO]). Mice were subcutaneously implanted with an RA pellet or mock pelleted by using a 10-gauge precision trochar (Innovative Research of America). Additional mouse experiments were carried with RA, GW0742, or TTNPB dissolved in sesame oil (Sigma Aldrich) and administered by direct pipetting (0.16 mg/50 μl) into the animals' mouths. Tissues were harvested after an overnight fast at the end of the experimental duration.

Body weights and food intake. Twenty-four-hour food consumption was measured once a week. During each 24-h period, mice were housed individually with a thin layer of bedding, and a known quantity of diet was provided. Food remaining after 24 h was weighed. Body weights were measured before and after the 24-h feeding period.

A glucose tolerance test was measured in mice fasted for 6 h and injected intraperitoneally with glucose (2 g/kg). Blood was sampled from the tail vein and at 0, 15, 30, 60, and 120 min using an UltraTouch meter.

Blood parameters. After an overnight fast, mice were killed, and blood was collected by a heart puncture. Plasma was isolated by using Microtainer plasma separator tubes (Becton Dickinson). Insulin was measured by using an ultrasensitive mouse insulin enzyme-linked immunosorbent assay (Mercodia, Lincoln Park, MO). Other parameters were measured by Veterinary Diagnostic Services (Marshfield Laboratories, Marshfield, WI).

Histology. Tissues were extracted and preserved in 10% formalin and then processed and sectioned by the Case Western Reserve University Histology Core. Sections were analyzed by using a Zeiss camera. Liver cell count was determined by counting single nuclei of individual cells in a ×10 field from two sections per mouse; four mice were counted from each group.

RESULTS

Adipogenesis is accompanied by downregulation of RARs and upregulation of PPARβ/δ signaling. To explore the mechanisms that underlie the involvement of RA in lipid homeostasis, we examined the signaling pathways by which the hormone functions in adipocytes. To this end, the well-established cultured adipocyte cell model NIH 3T3-L1 was used. Preadipocyte cells were allowed to grow for 2 days after confluence and induced to differentiate using a standard hormone mix (10 μg of insulin/ml, 0.5 mM IBMX, 0.25 mM dexamethasone). Three days later, the medium was replaced with DMEM supplemented with 10% FBS, and cells were grown for 4 days. Differentiation was verified by monitoring lipid accumulation and by examining the induction of the adipocyte marker FABP4 (see Fig. S1a and b in the supplemental material).

The expression levels of the RARs and PPARβ/δ and their respective cognate iLBPs, CRABP-II and FABP5, were assessed in preadipocytes and in differentiated cells. In agreement with a previous report (44), the data showed that preadipocytes express two RAR isotypes, RARα and RARγ, and that both of these are downregulated upon cell differentiation (Fig. 1a). The expression of CRABP-II, the iLBP that delivers RA to RARs, also markedly decreased during differentiation (Fig. 1b and c). These observations suggest that RARs can be

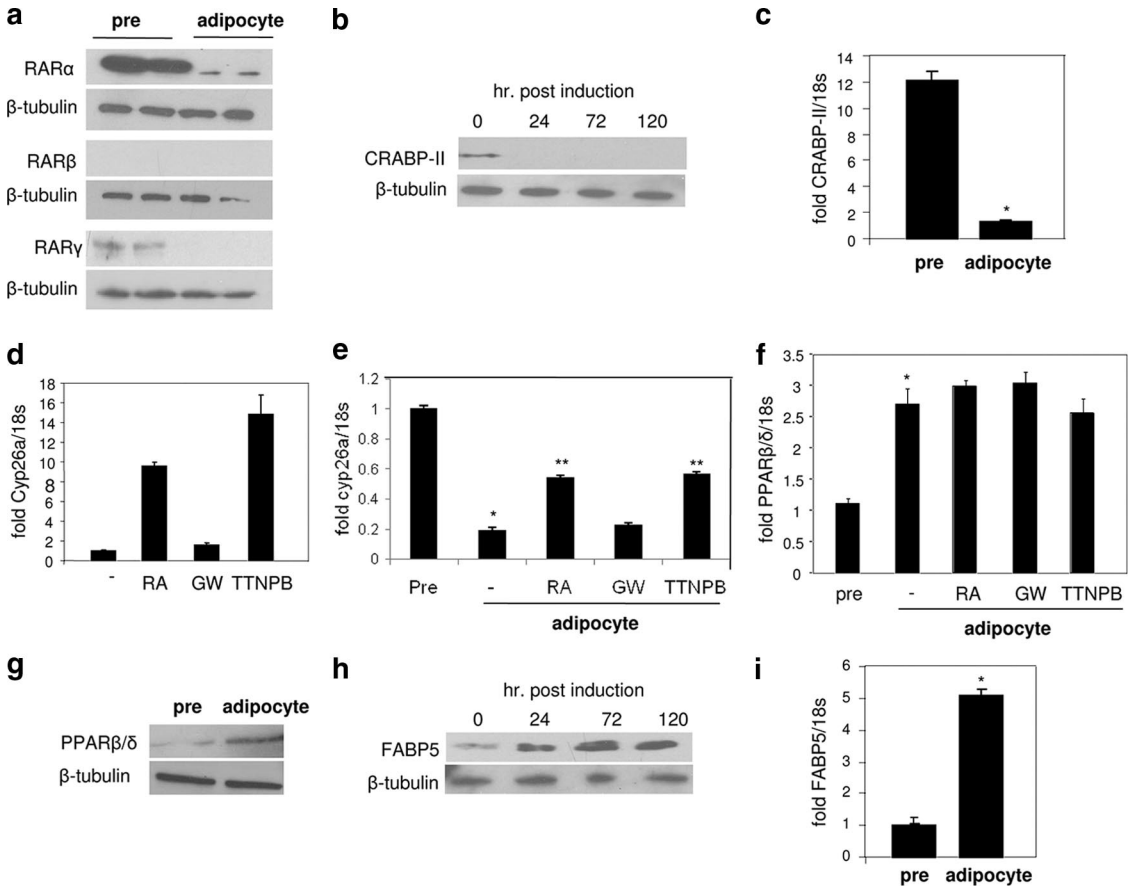

FIG. 1. Expression of RA binding proteins and receptors during adipocyte differentiation. (a) Immunoblots of RARα, RARβ, and RARγ in 3T3-L1 cell prior to (pre) and after (adipocyte) differentiation. (b) Immunoblot of CRABP-II in preadipocytes (lane 0) and at indicated times after differentiation induction. (c) Levels of CRABP-II mRNA in preadipocytes and differentiated adipocytes. (d and e) Levels of mRNA of Cyp26a in preadipocytes (d) and in differentiated adipocytes (e) treated with denoted ligands (0.1 μM, 6 h). (f) Level of PPARβ/δ mRNA in preadipocytes and differentiated adipocytes treated with the denoted ligands (0.1 μM, 6 h). (g) Immunoblot of PPARβ/δ in preadipocytes and differentiated adipocytes. (h) Immunoblot of FABP5 expression in preadipoctyes (lane 0) and at the indicated times after differentiation induction. (i) Level of FABP5 mRNA in preadipocytes and differentiated adipocytes. *, $P < 0.01$; **, $P < 0.05$ (versus preadipocytes).

activated in preadipose cells and that its potential activation decreases in the mature adipocyte. In support of this conclusion, expression of one of the hallmarks of RAR activation, the direct target gene encoding Cyp26a, was induced by >10-fold upon treatment of preadipocytes with either the synthetic pan-RAR ligand, TTNPB, or RA (Fig. 1d). The expression of the Cyp26a gene was markedly reduced in differentiated adipocytes (Fig. 1e). This gene could be induced by RA and TTNPB in mature cells but, as expected considering the low expression levels of CRABP-II and RARs in these cells, the induction was subdued and did not restore Cyp26a expression to the level found in preadipocytes (Fig. 1e). In contrast, the expression levels of the alternative RA receptor, PPARβ/δ, and its cognate iLBP, FABP5, were significantly higher in differentiated adipocytes than in precursor cells (Fig. 1f to i). The expression of PPARβ/δ, FABP5, and CRABP-II were not affected by treatment with RA, the synthetic selective ligand for PPARβ/δ GW0742, or the RAR ligand TTNPB (Fig. 1f and see Fig. S1c to f in the supplemental material).

To examine the transcriptional activity of the FABP5/PPARβ/δ pathway in mature adipocytes, the expression of various genes that were reported to be regulated by this receptor was examined. Special attention was given to genes that encode for proteins involved in lipid and sugar homeostasis: ADRP, a structural protein associated with lipid droplets (40); uncoupling proteins 1 and 3 (UCP1 and UCP3), involved in energy dissipation and fatty acid oxidation (41); ALDH9, a key enzyme in the generation of carnitine and thus in fatty acid oxidation; and ANGPTL4, a secreted adipose factor that plays essential roles in lipid and glucose metabolism (15, 20). The expression of mRNAs for two other PPARβ/δ target genes involved in insulin signaling was evaluated: the PDK1 gene (9) and the insulin-regulated glucose transporter GLUT4 gene (6). As shown in Fig. 2, the mRNA levels for all of these genes were higher in mature versus precursor adipocytes. The expression of these genes was induced upon treatment with RA or the synthetic PPARβ/δ-selective ligand GW0742 in both preadipocytes and mature adipocytes but, with the exception of UCP1, the induction was greater in the mature cells. The RAR ligand TTNPB did not affect the expression of PDK1, ADRP, UCP3, GLUT4, or ANGPTL4. However, expression of UCP1 and ALDH9 was induced both by GW0742 and by the RAR

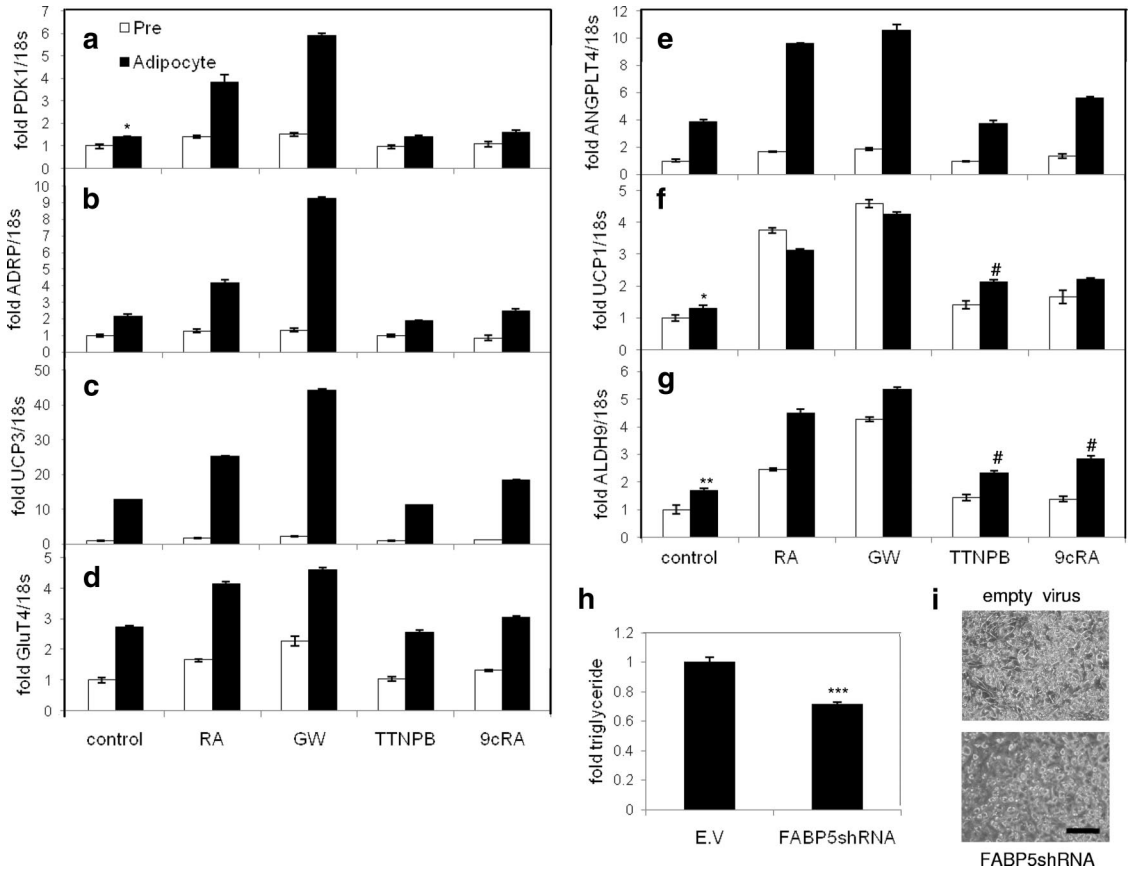

FIG. 2. PPARβ/δ target genes are induced upon adipocyte differentiation and in response to RA. (a to g) Levels of denoted PPARβ/δ target genes in preadipocytes (Pre) and in differentiated (Adipocyte) adipocytes treated with RA, the PPARβ/δ-selective ligand GW0742, or the RAR-selective ligands TTNPB or 9cRA (0.1 μM, 4 h) (*, $P = 0.05$; and **, $P = 0.01$ [versus preadipocytes]; #, $P = 0.01$ [versus untreated control]). (h and i) FABP5 is involved in adipocyte differentiation. Preadipocytes were infected with a lentivirus harboring FABP5shRNA or an empty virus (E.V) and then induced to differentiate. (h) Triglyceride assays were carried out upon completion of differentiation indicating that the decreased FABP5 levels resulted in a lower triglyceride content (***, $P < 0.001$ [versus empty virus]). (i) Cells were observed under normal phase microscopy (×10 magnification). Adipocytes that differentiated under low FABP5 levels displayed fewer lipid droplets.

ligand TTNPB (Fig. 2f and g), suggesting that the associated genes are regulated by both RAR and PPARβ/δ. These observations are in agreement with the reports that *UCP1* comprises a direct target gene for both of these receptors (1, 35, 38). Treatment with 9cRA slightly induced the expression of some of the genes, but it did not recapitulate the effects of RA. Hence, RA did not function through activation of RXR.

Taken together, the data indicate that RA can activate both of its receptors in preadipocytes, as well as in mature adipocytes, but that adipogenesis is accompanied by a shift in the balance between the receptors, decreasing CRABP-II/RAR activities and enabling efficient activation of the FABP5/PPARβ/δ pathway. To examine the importance of the upregulation of FABP5 for the process of adipocyte differentiation, the level of this protein was decreased by infecting preadipose cells with a lentivirus harboring FABP5 shRNAs. Cells were treated with a differentiation mixture, and their state of differentiation was examined 7 days later. Immunoblots verified that FABP5 expression in cells infected with the shRNA was markedly reduced at the end of the experiment (see Fig. S1g in the supplemental material). Inhibition of the upregulation of FABP5, which accompanies normal adipogenesis, resulted in incomplete differentiation, as reflected by a lower triglyceride content of these cells (Fig. 2 h and i). Hence, FABP5, likely through its cooperation with PPARβ/δ, is an important component of the adipocyte differentiation program.

In mature adipocytes, RA-induced, PPARβ/δ-mediated activities cross talk with insulin signaling. FABP5 and PPARβ/δ were previously shown to cooperate in mediating the transcriptional activities of RA in keratinocytes and in mammary carcinoma cells (32, 33). To examine whether these proteins also cooperate in RA activities in adipocytes, the effects of decreasing their expression on the ability of RA to induce PPARβ/δ target genes were monitored. FABP5 or PPARβ/δ were downregulated by infecting differentiated adipocytes with lentiviruses harboring the corresponding shRNAs (see Fig. S1h and i in the supplemental material). Decreasing the expression of either the binding protein or the receptor hampered the ability of both the synthetic PPARβ/δ ligand GW0742 and RA to induce the expression of various PPARβ/δ target genes, including *PDK1* (Fig. 3a and b; see also Fig. S2a to f and h in the supplemental material). In contrast, reducing the expression of FABP5 or PPARβ/δ did not decrease the expression of the

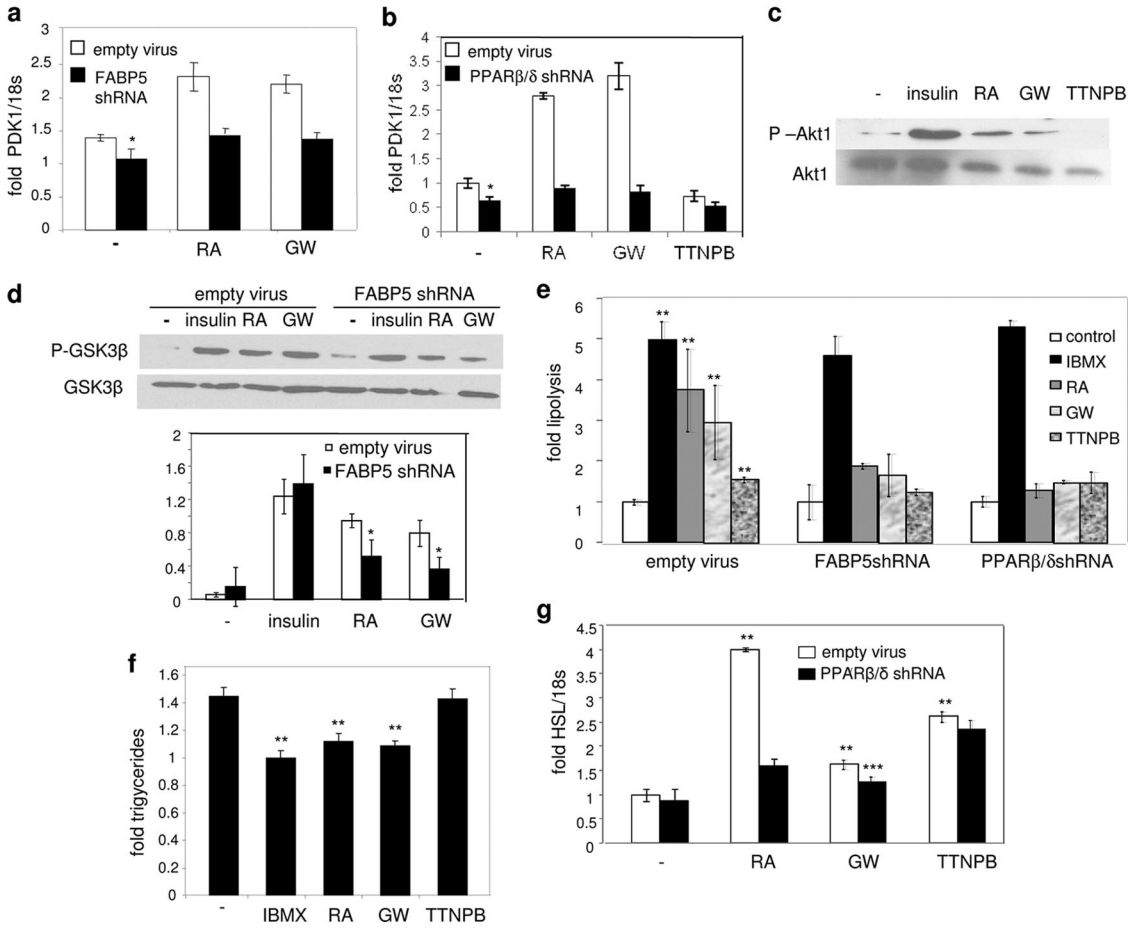

FIG. 3. sIn adipocytes, RA induces PPARβ/δ target genes in an FABP5- and PPARβ/δ-dependent manner, activates Akt signaling, and enhances lipolysis. (a and b) PDK1 mRNA in the absence or presence of FABP5 shRNA or PPAR-β/δ shRNA (*, $P < 0.01$ [versus empty virus controls]). (c) Immunoblots of total and phosphorylated Akt1 in differentiated adipocytes treated with insulin (5 nM, 20 min) or the denoted ligands (0.1 μM, 12 h). Experiments were repeated three times with similar results. (d) The top panel shows representative immunoblots of total and phosphorylated GSK3β in differentiated adipocytes infected with a control lentivirus or a lentivirus harboring FABP5shRNA and treated with insulin (5 nM, 30 min) or the denoted ligands (0.1 μM, 16 h). The bottom panel shows the quantitation of effects of insulin and ligands on GSK3β phosphorylation (mean ± standard deviation, $n = 3$). *, $P < 0.05$ (versus corresponding empty virus controls). (e) Differentiated adipocytes were infected with a control lentivirus or lentivirus harboring FABP5 shRNA or PPARβ/δ shRNA, treated with the denoted ligands (0.1 μM, 24 h), and lipolysis assays were carried out. **, $P < 0.05$ (versus untreated control). (f) Triglyceride levels in mature adipocytes treated with denoted ligands as in panel e. **, $P < 0.05$ (versus untreated control). (g) Levels of mRNA of HSL in adipocytes infected with empty lentivirus or virus harboring PPARβ/δ shRNA for 5 days and then treated with the denoted ligands (0.1 μM, 4 h.). **, $P < 0.05$ (versus untreated empty virus). ***, $P = 0.05$ (versus GW0742-treated empty virus).

RAR target gene encoding Cyp26a (see Fig. S2g and i in the supplemental material).

PDK1 is a downstream effector of phosphatidylinositol 3-kinase that mediates some of the actions of insulin, most notably by activation of PKB/Akt1. In turn, Akt1 phosphorylates and thus inactivates GSK3β. The observed RA-induced, FABP5- and PPARβ/δ-mediated induction of PDK1 suggests that a cross talk exists between RA and insulin signaling. Indeed, treatment of adipocytes with insulin, or with either RA or GW0742, induced the phosphorylation of both Akt1 (Fig. 3c) and GSK3β (Fig. 3d). Similarly to other aspects of PPARβ/δ signaling, decreasing the expression of FABP5 inhibited the ability of RA and GW0742 to induce GSK3β phosphorylation (Fig. 3d). Taken together with the observations that RA induces the expression of GLUT4 (Fig. 2d), the data suggest that RA supports insulin action in adipocytes. It may be worth noting in regard to this that it has indeed been reported that PPARβ/δ agonists stimulate glucose uptake in myocytes (16, 17).

RA-induced activation of PPARβ/δ and RARs in adipocytes enhances lipid oxidation and depletes lipid stores. The effects of RA on lipid hydrolysis and on triglyceride accumulation in adipocytes were then investigated. IBMX, a cyclic nucleotide phosphodiesterase inhibitor, induces adipocyte differentiation when administered to preadipocytes. In mature adipocytes, this compound triggers the phosphorylation and activation of HSL, an enzyme that plays a major role in catalyzing lipolysis in adipocytes (43). Hence, in mature adipocytes, IBMX functions as a potent inducer of lipolysis and was thus used as a positive control. IBMX, RA, and GW0742 all triggered lipolysis (Fig. 3e). The RAR ligand TTNPB also induced lipolysis, although to a smaller extent (Fig. 3e). Induction of lipolysis by RA and GW0742, but not by IBMX, was inhibited upon downregulation of either FABP5 or PPARβ/δ (Fig. 3e). In accor-

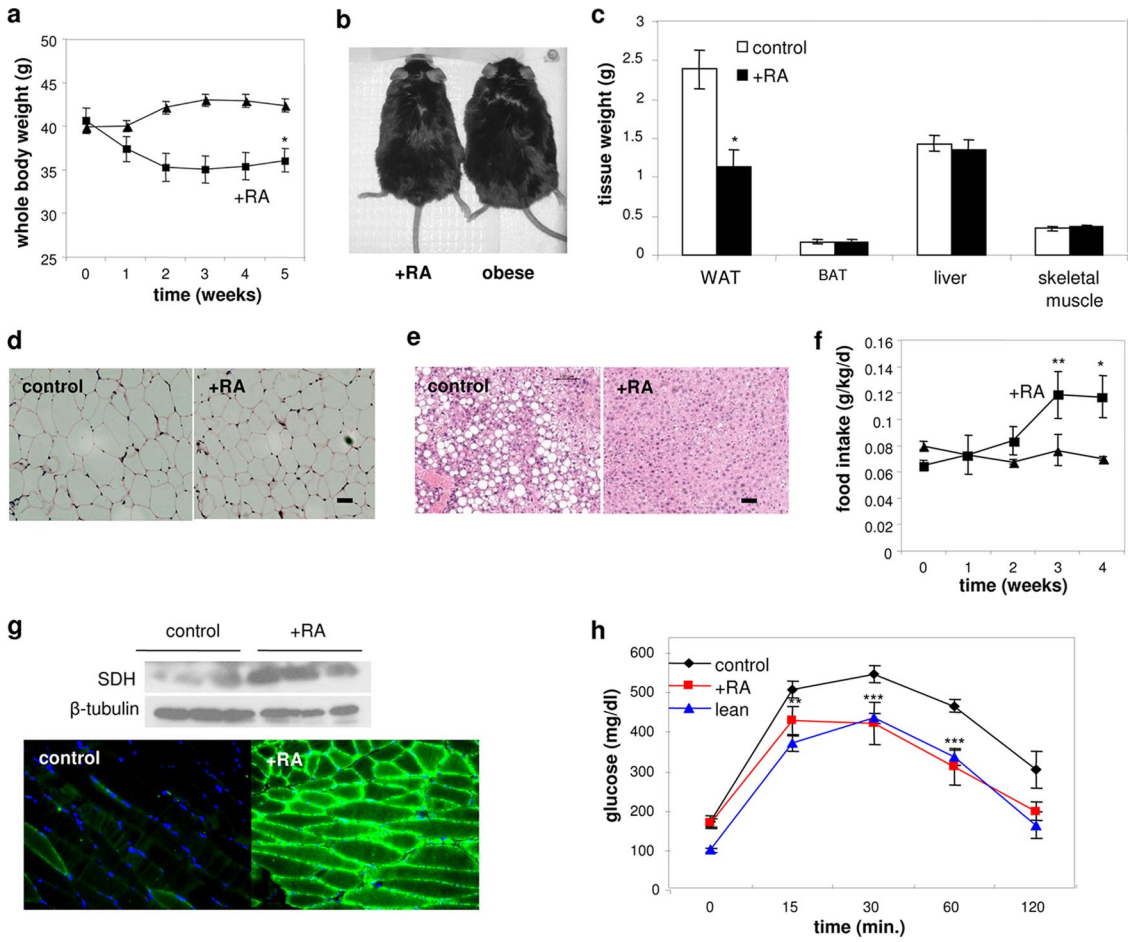

FIG. 4. Effects of RA treatment on obese mice fed a high-fat/high-carbohydrate diet. (a) Whole-body weight of obese mice ($n = 16$) and obese mice treated with RA ($n = 14$; *, $P < 0.001$). (b) Dorsal view of obese mice and obese mice treated with RA for 5 weeks. (c) Tissue weights of epididymal/abdominal WAT, brown adipose tissue (BAT), gastronemius and soleus skeletal muscle, and livers of obese mice ($n = 9$) and mice treated with RA for 5 weeks ($n = 7$; *, $P < 0.001$ [versus untreated mice]). (d and e) Hematoxylin and eosin staining of cross-sections of WATs (d) and livers (e) of untreated and RA-treated mice. Tissues were preserved in 10% formalin. Samples were embedded in paraffin, sectioned, and stained at the Pathology Core Facility of Case Western Reserve University. Bar, 100 mm. (f) Food intake of untreated and RA-treated obese mice (*, $P < 0.001$; **, $P = 0.05$ [versus untreated animals]). (g) The mitochondrial marker SDH in gastronemius muscle after a 5-week RA visualized by immunoblots (top) and by immunofluorescence microscopy (bottom). Photographs were taken at a magnification of ×20. (h) Glucose tolerance test in obese mice ($n = 7$), RA-treated obese mice ($n = 7$), and lean control mice ($n = 4$). **, $P = 0.05$; ***, $P = 0.005$ (versus untreated obese mice).

dance with the markedly enhanced lipolysis in response to RA or GW0742, these ligands reduced the triglyceride content of the cells (Fig. 3f). Removal of the ligands from growth media restored the triglyceride content (see Fig. S3a in the supplemental material), indicating that their effects are reversible. To further examine the molecular mechanism that underlies lipid hydrolysis, the effects of RA, GW0742, and TTNPB on the expression of HSL were monitored. HSL expression was induced upon a 4-h treatment with either GW0742 or TTNPB, and treatment with RA resulted in a higher induction than that of either of the synthetic ligands alone (Fig. 3g). Downregulation of PPARβ/δ markedly decreased the ability of RA to induce the expression of HSL but did not abolish it (Fig. 3g). These observations imply that, in adipocytes, HSL is regulated by both RARs and PPARβ/δ and that the induction of lipolysis by RA is therefore mediated by both of its receptors. Signaling through PPARβ/δ appears to comprise a major component of this activity.

RA suppresses adiposity and improves insulin responsiveness in vivo. We then set out to investigate whether RA activates PPARβ/δ and RARs in vivo and to examine the physiological outcomes of these activities. We used a mouse model of diet-induced obesity and insulin resistance. C57BL/6Ntac male mice were fed a high-fat/high-sucrose diet for 16 weeks prior to experimentation. At the beginning of experiments, these mice weighed ~1.5-fold more than a cohort fed a standard diet. The obese mice displayed an elevated blood insulin level (lean mice, 0.35 ± 0.04 μg/liter; obese mice, 2.21 ± 0.60 μg/liter [mean ± the standard error of the mean, $n = 16$]) and a sluggish response in glucose tolerance tests (Fig. 4h). Obese mice were separated into two groups. Both groups were continually maintained on a high-fat/high-sucrose diet, and one of them was systemically treated with RA by implanting slow-release RA pellets (15 mg, 90-day release; Innovative Research of America) subcutaneously. A 5-week RA treatment resulted in loss of ~15% body weight (Fig. 4a and b). This weight loss

stemmed mainly from reduced abdominal and epididymal white adipose tissue (WAT) mass, which decreased by >2-fold (Fig. 4c) and was accompanied by a decrease in adipocyte cell size (Fig. 4d). In addition, hepatic steatosis, reflected by lipid accumulation in livers of the obese mice, was completely reversed upon RA treatment (Fig. 4e). The lack of change in total liver weight despite RA-induced depletion of hepatic lipids can be readily understood considering that, even under conditions of steatosis, lipids comprise only 3 to 4% of liver weight, and taking into account the regenerative capacity of this organ (see Fig. S3b in the supplemental material).

The food consumption of RA-treated mice was higher than that of the untreated cohort (Fig. 4f), suggesting that the reduced adiposity stemmed from a higher rate of energy utilization rather than a decreased intake. RA treatment also resulted in increased expression of muscle SDH, indicating an elevated mitochondrial content in skeletal muscle (Fig. 4g). Taken together, the data suggest that RA increased metabolic activities in the mice and that it did so by enhancing lipid hydrolysis, fatty acid oxidation, and energy dissipation, as well as by inducing mitochondrial proliferation in muscle. In agreement with the notion that RA induced both lipolysis and fatty acid oxidation, the plasma levels of free fatty acids were similar in RA-treated and untreated mice, but the plasma concentration of glycerol was elevated in the treated cohort (untreated mice, 143.3 ± 0.8 μM; RA-treated mice, 192.0 ± 0.9 μM [mean ± the standard error of the mean, $n = 8$]). In addition, while the basal level of glucose was not altered upon RA treatment, glucose tolerance tests (Fig. 4 h) showed that both the peak and the rate of decrease of serum glucose levels after glucose injection into RA-treated mice resembled the response of the lean cohort, reflecting a marked improvement in the insulin sensitivity of the animals.

PPARβ/δ signaling in vivo is downregulated upon the onset of obesity and is reactivated by RA. To further examine the basis of the effects of RA on energy homeostasis in the mice, the expression levels of RARs and PPARβ/δ, were assessed. mRNA for all three RAR isotypes were found to be expressed in mouse adipose tissue, and their levels in obese mice were similar to those observed in their lean counterparts (Fig. 5a). Interestingly, the expression of both PPARβ/δ and FABP5 was markedly lower in obese mice than in lean mice (Fig. 5b and c), suggesting that obesity is associated with repression of PPARβ/δ signaling. In accordance, adipose tissue of obese mice expressed lower levels of the direct PPARβ/δ target genes *PDK1* and *UCP3* (Fig. 5d). Obese animals also displayed a trend suggesting a lower level of phosphorylation of Akt1 (Fig. 5e) compared to their lean counterparts. RA treatment induced the expression of the direct RAR target gene RARβ in adipose tissue (Fig. 5a) and of various PPARβ/δ target genes in adipose tissue and in muscle (Fig. 5d). RA also increased Akt1 phosphorylation in adipose tissue (Fig. 5e). Hence, similarly to the responses of cultured adipocytes, RA activates both RARs and PPARβ/δ in adipose tissue in vivo. Interestingly, treatment of obese mice with RA also restored the expression of both PPARβ/δ and FABP5 to levels similar to those found in lean animals (Fig. 5b and 5c).

Activation of FABP5/PPARβ/δ signaling upon RA treatment may be a direct effect, or it can indirectly result from other physiological activities of RA, such as the induction of weight loss. To address this issue, the effects of a short-term RA treatment were investigated. Mice were implanted with slow-release RA pellets for 2 days, a time frame in which no weight loss was observed. Examination of gene expression profiles showed that, similarly to the long-term treatment, a 2-day RA treatment resulted in a marked induction of multiple PPARβ/δ target genes in adipose tissue and muscle (Fig. 5f). Activation of PPARβ/δ by RA thus appears to be the cause rather than the consequence of weight loss. In contrast, a 2-day RA treatment had no effect on the expression of either PPARβ/δ or FABP5 (data not shown), indicating that the recovery of the expression of these genes is a secondary event. The molecular basis for the downregulation of these proteins in obese animals and of their recovery upon RA treatment has not yet been determined.

In vivo activities of RA are mediated by both RARs and PPARβ/δ. To distinguish between activities of RA that are mediated by PPARβ/δ versus those mediated by RARs, the effects of treating obese mice with RA were compared to treatment with the synthetic PPARβ/δ-selective ligand GW0742. In these experiments, since slow-release pellets of GW0742 are not available, ligands were dissolved in sesame oil and administered to mice by oral feeding (0.16 mg/day). Mice were treated for 3 weeks. RA and GW0742 similarly induced the expression of the adipose PPARβ/δ target genes *PDK1*, *ADRP*, *ANGPTL4*, *UCP3*, and *ALDH9* (Fig. 6a). Interestingly, in accordance with the observations that the expression of *UCP1* and *HSL* is regulated by both PPARβ/δ and RARs (Fig. 2f and 3g), these genes were upregulated to a markedly greater extent upon administration of RA versus the administration of GW0742 (Fig. 6a). The two ligands similarly affected various whole-body parameters. Thus, food intake was elevated upon administration of either RA or GW0742 (Table 1), suggesting that both ligands increased energy expenditure. In agreement with this notion, the body temperatures of mice treated with either compound were higher than those of the control group (Table 1). Both RA and GW0742 increased the plasma concentrations of glycerol without affecting the levels of free fatty acids, reduced the blood levels of triglycerides and insulin (Table 1), and upregulated the expression of adipose PPARβ/δ and FABP5 (Fig. 6b).

Some differences between the RA- and the GW0742-treated groups were observed. RA was notably more effective in inducing weight loss and in reducing insulin and triglyceride levels (Table 1). These differential effects may be readily understood in view of the observations that RA was more efficient in inducing the expression of adipose HSL and UCP1 (Fig. 6a). In addition, treatment with GW0742, but not with RA, resulted in an elevated level of blood cholesterol (Table 1). Elevation of plasma cholesterol upon administration of a PPARβ/δ-selective ligand to mice was previously reported and it was shown to originate from higher levels of high-density lipoprotein particles (HDL) and HDL-associated cholesterol (19, 29). This effect was attributed to PPARβ/δ-induced upregulation of hepatic apo A1, the major protein in HDL (19, 29). In agreement with these reports, treatment of obese mice with GW0742 markedly upregulated hepatic apo A1 mRNA. In contrast, RA treatment only slightly affected apo A1 expression (Fig. 6a). A rationale for the discrepancy between the effects of RA and GW0742 in the context of this gene is

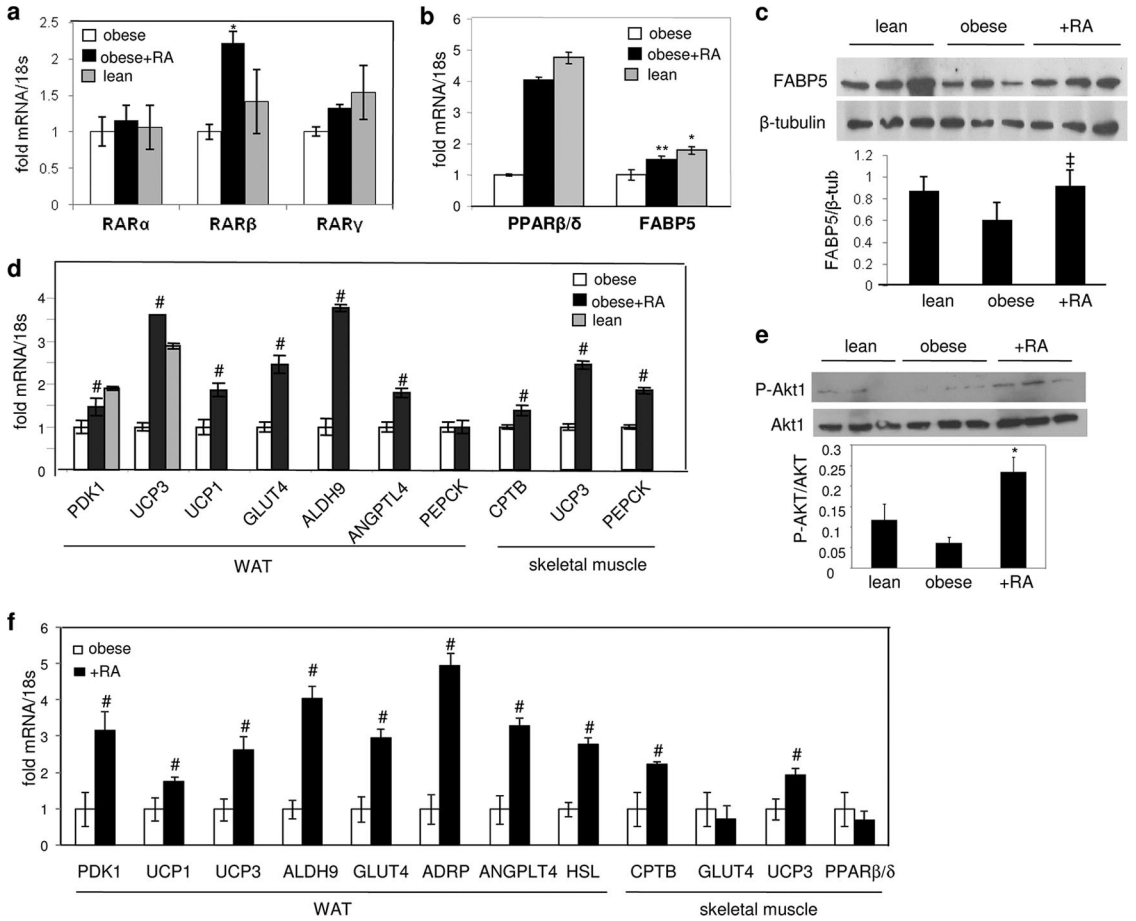

FIG. 5. Expression of RARs, PPARβ/δ, FABP5, and PPARβ/δ target genes in lean, obese, RA- and GW0742-treated mice. (a and b) Levels of mRNA for denoted RARs (a) and PPARβ/δ and FABP5 (b) in the adipose tissues of lean, obese, and RA-treated obese mice ($n = 3$ per group; *, $P < 0.005$; **, $P = 0.04$ [versus lean mice]). (c) The top panel shows immunoblots of FABP5 in the adipose tissues of lean, obese, and RA-treated mice. Each lane represents an individual mouse. The bottom panel shows the quantitation of the immunoblots in the top panels (‡, $P = 0.07$ [versus untreated obese mice]). (d) Levels of mRNA for the denoted genes in WAT and skeletal muscles of lean, obese, and obese mice treated with RA for 5 weeks ($n = 3$; #, $P = 0.05$ [versus untreated obese mice]). (e) The top panel shows immunoblots of total and phospho-Akt1 in WAT of lean, obese, and mice treated with RA for 5 weeks. The bottom panel shows the quantitation of the immunoblots in the top panels (*, $P < 0.005$ [versus obese mice]). (f) Levels of mRNA for the denoted PPARβ/δ target genes in adipose tissues and skeletal muscles of untreated mice and mice treated with RA for 2 days. #, $P = 0.05$ (versus untreated obese mice).

suggested by the report that activation of RARs repressed apo A1 expression in cynomolgus monkey hepatocytes (27). To dissect between RAR and PPARβ/δ activities, the effects RA on gene expression were compared to those of the PPARβ/δ ligand GW0742 and the RAR ligand TTNPB. Mice were fed with ligands for 2 days at 0.16 mg/day, WATs and livers were harvested, and RNA was isolated. The expression levels of adipose HSL and UCP1 and of hepatic apo A1 were measured by Q-PCR. The expression of the HSL and UCP1, which are targeted by both RA receptors, was induced by both GW0742 and TTNPB, as well as by RA. In contrast, the expression of apo A1 was efficiently induced by GW0742 but somewhat downregulated upon treatment with TTNPB (Fig. 6c). The data thus suggest that the subdued response of this gene to RA treatment may reflect that activation of RARs by the hormone inhibits PPARβ/δ-mediated induction. To further examine this notion, the effects of RA, GW0742, TTNPB, or a mixture of GW0742 and TTNPB on the expression of apo A1 in HepG2 human liver carcinoma cells were examined. Cells were treated with ligands for 4 h, RNA was isolated, and apo A1 mRNA was measured by Q-PCR. As shown in Fig. 6d, similar to the in vivo responses, GW0742 efficiently upregulated apo A1, TTNPB somewhat decreased the basal expression level of the gene, and RA treatment resulted in a small response. Importantly, a mixture of GW0742 and TTNPB resulted in a response that was all but identical to that of RA, demonstrating that activation of RARs inhibits PPARβ/δ-mediated induction of apo A1. These observations provide a rationale for the observations that, while treatment of mice with a PPARβ/δ selective ligand elevates blood cholesterol (19, 29) (Table 1), treatment with RA does not.

DISCUSSION

Several lines of evidence demonstrated that vitamin A is involved in the regulation of adiposity and energy balance. Hence, it was reported that administration of vitamin A to mice leads to weight loss (11), that genetic manipulation of

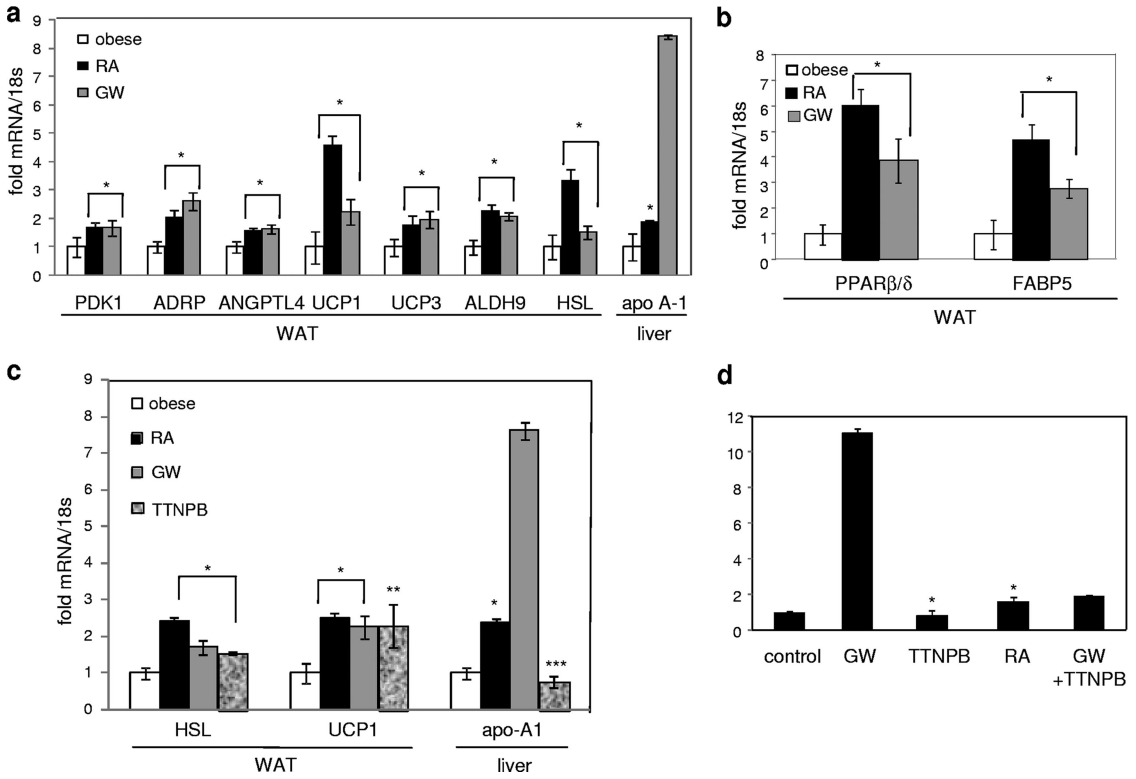

FIG. 6. Effects of RA, GW0742, and TTNPB on gene expression in obese mice and in HepG2 cells. (a and b) Levels of mRNA of PPARβ/δ target genes (a) and of PPARβ/δ and FABP5 (b) in untreated, RA-treated, and GW0742-treated mice after 3 weeks of treatment (0.16 mg/day). *, $P \leq 0.05$ (versus obese untreated mice). (c) Levels of adipose HSL and UCP1 and hepatic apo A1 in obese mice fed sesame oil (obese), RA, GW0742, or TTNPB for 2 days (0.16 mg/day). WATs and livers were harvested, RNA was isolated, and mRNA levels were examined by Q-PCR. *, $P \leq 0.05$; **, $P = 0.09$; ***, $P = 0.06$ (versus obese untreated mice). (d) Levels of apo A1 mRNA in HepG2 cells treated with the denoted ligands (0.1 μM, 4 h). *, $P \leq 0.05$ (versus untreated mice).

enzymes that mediate vitamin A metabolism in mice result in alterations in adiposity (45, 46), and that treatment of rodents with vitamin A or RA can change the expression levels of adipose genes involved in energy homeostasis (11, 25). However, the molecular mechanisms that underlie these effects remained incompletely understood.

TABLE 1. Characteristics of obese mice treated with RA and GW0742 for 3 weeks

Parameter	Mean ± SD for each mouse treatment group[a]		
	Control mice	RA-fed mice	GW0742-fed mice
General			
wt loss (%)	5 ± 0.2	11 ± 0.4*	3 ± 0.1
Food intake (treated/control)	1	1.33 ± 0.05**	1.18 ± 0.04*
Rectal temp (°C)	36.7 ± 0.2	37.2 ± 0.2**	37.1 ± 0.1**
Concn in blood			
β-Hydroxybutyrate (mg/dl)	3.8 ± 0.3	3.9 ± 0.3	3.9 ± 0.3
Cholesterol (mg/dl)	200.3 ± 8.4	181.8 ± 5.8	229 ± 8***
Triglycerides (mg/dl)	185.8 ± 18.1	94 ± 16**	141.3 ± 14.1‡
Free fatty acids (mM)	0.50 ± 0.04	0.41 ± 0.05	0.46 ± 0.04
Glycerol (μM)	70.0 ± 0.3	92.0 ± 0.5*	90.0 ± 0.4*
Insulin (μg/liter)	4.4 ± 0.5	2.0 ± 0.4***	3.4 ± 0.3

[a] Sixteen-week-old mice fed a high-fat/high-sucrose diet were orally fed RA or GW0742 (0.16 mg/day) daily for 3 weeks. Blood was collected after an overnight fast. Plasma was isolated by using Microtainer plasma separator tubes (Becton Dickinson). Insulin was measured by using an ultrasensitive mouse insulin enzyme-linked immunosorbent assay (Mercodia). Other parameters were measured by Veterinary Diagnostic Services (Marshfield Laboratories). Data are means from eight animals. *, $P = 0.05$; **, $P < 0.005$; ***, $P = 0.02$; ‡, $P = 0.07$ (versus untreated mice).

The observation that, in addition to activating its classical nuclear receptors, RARs, RA can function as a ligand for PPARβ/δ (32, 33, 37) suggests that the spectrum of the biological functions of this hormone is wider than previously suspected. An important function of the alternate RA receptor, PPARβ/δ, is its ability to regulate lipid and sugar homeostasis in various tissues, including the adipose tissue (2, 3, 18). We show here that the expression levels of RARs and their cognate lipid-binding protein, CRABP-II, are downregulated and that the expression levels of proteins that mediate the alternate RA-induced pathway, i.e., PPARβ/δ and FABP5, markedly increase upon adipocyte differentiation. Consequently, RA can activate both RAR and PPARβ/δ in both preadipocytes and in differentiated adipose cells, but the balance of RA signaling shifts upon adipocyte differentiation, reducing CRABP-II/RAR activities and enabling efficient FABP5/PPARβ/δ activation. It is interesting in regard to these observations that it has been reported that activation of RARs in the early stages of adipogenesis inhibits differentiation (34). Hence, downregulating RAR activity and diverting RA to PPARβ/δ appears to be a critical component of the differentiation process. In accordance with this conclusion, we show that knocking down FABP5 expression resulted in incomplete adipocyte differentiation (Fig. 2h and i). These observations are in agreement with the reports that the receptor associated with FABP5,

PPARβ/δ, is a necessary component for adipocyte differentiation, as demonstrated by the observations that PPARβ/δ-null mice display reduced adipose tissue (2).

Using cultured cells and a high-fat/high-carbohydrate diet-induced mouse model of obesity, we demonstrate that RA evokes multiple aspects of the program known to be triggered upon activation of PPARβ/δ. In adipocytes, RA induced the expression of PPARβ/δ target genes, including genes involved in lipid metabolism—e.g., genes encoding uncoupling proteins, ALDH9, required for fatty acid oxidation, and ANGPTL4, an adipokine that regulates plasma lipoprotein metabolism (20)—and genes encoding proteins such as PDK1 and GLUT4, which are involved in insulin responses. In vivo, RA treatment upregulated the expression of lipid- and sugar-processing PPARβ/δ target genes in adipose tissue and liver, and it recapitulated the reported activity of PPARβ/δ in increasing skeletal muscle mitochondrial content (42). Remarkably, RA treatment of obese mice led to weight loss and to an improved glucose tolerance despite the larger food intake of the treated mice. Taken together, with the higher body temperature of RA-treated mice, these observations indicate that the weight loss originated from enhanced energy utilization.

In further support of the notion that the beneficial effects of RA were to a large extent mediated by PPARβ/δ, the in vivo effects of the hormone were similar to those of a synthetic selective ligand for this receptor. Thus, both RA and GW0742 induced the expression of adipose PPARβ/δ target genes, enhanced food intake, raised the body temperature, increased the plasma concentrations of glycerol without affecting levels of free fatty acids, reduced the blood levels of triglycerides and insulin, and upregulated the expression of adipose PPARβ/δ and FABP5. Interestingly, although similar trends were observed, RA was more effective than GW0742 in inducing weight loss and in reducing insulin and triglyceride levels. These findings may be understood in view of the observations that some genes involved in lipid hydrolysis and fatty acid oxidation, such as *ALDH9*, *UCP1*, and *HSL* (see Fig. 2, 3g, and 6a), are regulated by both PPARβ/δ and RARs. Hence, while a major component of the protective activities of RA against adiposity and insulin resistance stems from activation PPARβ/δ, the heightened efficacy of this ligand likely originates from its ability to also activate RARs. RA-induced activation of both RARs and PPARβ/δ also underlies the observations that treatment of obese mice with GW0742 but not with RA results in elevated plasma cholesterol. Hence, while PPARβ/δ induces the expression of apo A1, RARs inhibit the effect (Fig. 6). Consequently, administration of a PPARβ/δ-selective ligand leads to higher blood levels of HDL cholesterol (19, 29), but activation of both RARs and PPARβ/δ by RA does not.

The expression levels of both FABP5 and PPARβ/δ were found to be markedly lower in obese mice (Fig. 5b), and they were restored to levels similar to those observed in lean mice after RA treatment. In accordance with these observations, it has been reported that FABP5 levels are lower in obese than in lean or weight-reduced human subjects (12). Deregulation of FABP5/PPARβ/δ signaling thus appears to be associated with the onset of obesity, and weight loss is correlated with reactivation of the pathway. The data indicate that the recovery of expression of the two proteins is a later indirect outcome of RA treatment. The mechanisms by which obesity downregulates these proteins and by which RA induces their recovery remain to be clarified. In an apparent discrepancy with the conclusion that downregulation of FABP5 is associated with obesity, it has been reported that FABP5-null mice are protected from diet-induced obesity and insulin resistance (22). It should be noted, however, that in this mouse model FABP5 was lacking throughout development. Since FABP5 and PPARβ/δ are closely involved in adipocyte differentiation, it is likely that the observed phenotype in the mice stems from impaired adipogenesis and thus reflects reduced or altered adipose tissue rather than reports on the functions of FABP5 in adipocytes that differentiate under normal conditions. Such an interpretation is supported by the observations that reducing the expression of FABP5 during adipogenesis resulted in incomplete differentiation (Fig. 2h and i).

The results of the present study show that RA treatment of obese mice leads to depletion of adipose lipid stores, induction of weight loss, reversal of hepatic steatosis, increase of muscle mitochondrial content, improvement of glucose tolerance, and reactivation of the FABP5/PPARβ/δ pathway. These data provide a rationale for the long-noted but poorly understood function of vitamin A in regulating energy balance, and they suggest that RA may be an efficacious agent in suppressing obesity and insulin resistance.

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